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Reconstitution of Bacterial Expressed Human CD94: The Importance of the Stem Region for Dimer Formation

Jeffrey C. Boyington,* Aisha N. Raiz,* Andrew G. Brooks,† Apisit Patamawenu,* and Peter D. Sun*,[1]

**Structural Biology Section, Office of The Scientific Director, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12441 Parklawn Drive, Rockville, Maryland 20852; and †Department of Microbiology and Immunology, University of Melbourne, Royal Parade, Parkville, 3052 Victoria, Australia*

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Human CD94 is a subunit of the disulfide-linked, heterodimeric natural killer (NK) cell surface receptor CD94/NKG2. This receptor, a member of the C-type lectin superfamily, participates in regulating NK cell directed lysis through interaction with the major histocompatibility antigen HLA-E. Two forms of CD94 were expressed using a bacterial expression system and refolded *in vitro*. One form, residues 34–179, designated S34, corresponds to the entire extracellular region of the receptor, including a 23-residue stem region, and the other, residues 51–179, designated E51, corresponds only to the putative carbohydrate recognition domain of the receptor. The refolded full-length S34 protein existed as a noncovalent dimer initially but formed an interchain disulfide bond upon storage for several months. In contrast, the stemless construct, E51, existed largely as a monomeric form. The stem region of S34, residues 34–56, is sensitive to proteolysis and its absence results in dissociation of the dimer. This suggests that the residues in the stem region of CD94 help to stabilize the dimeric conformation. © 2000 Academic Press

Elucidation of the mechanism by which natural killer (NK) cell cytotoxic activity and cytokine production are regulated has been a focal point in immunology in recent years. Several members of class I major histocompatibility complex (MHC) recognizing cell surface receptors on NK cells have been implicated in this regulatory process and identified as belonging to either the immunoglobulin or the C-type lectin-like superfamilies (1,2).

Human CD94, a type II transmembrane C-type lectin-like glycoprotein, is a component of the MHC class I recognizing receptor CD94/NKG2 expressed on the surface of NK cells which regulates cytotoxic killing (3,4). The functional form of this heterodimeric receptor has been shown to consist of an invariant CD94 polypeptide disulfide-linked to either NKG2A, -B, -C, or -E (5–8). The CD94 and NKG2 polypeptide chains each consist of a cytoplasmic tail (only 10 residues long in CD94), a transmembrane region, an extracellular stem region, and a C-terminal, extracellular C-type lectin-like domain. CD94/NKG2A/B and -C receptors are known to recognize the human nonclassical class I MHC molecule HLA-E as their ligand (9–12). More recently, genes for CD94 and NKG2 proteins have also been identified in mice and rats (13–18), and similar to its human counterpart, murine CD94/NKG2A recognizes a murine nonclassical class I MHC molecule, Qa-1 (19). It has been shown that the cell surface expression of NKG2A/B, -C, and -E proteins depends on the expression of CD94, whereas CD94 can be found on the surface of transfected cells without NKG2 (5,7). This suggests that, contrary to CD94, these NKG2 proteins do not exist in a stable form by themselves and must interact with a CD94 partner.

In murine systems, there is another family of receptors, comprising Ly-49A through Ly-49H, that have been identified as class I MHC recognizing homodimeric NK cell surface receptors also belonging to the C-type lectin-like superfamily (20–25). Characterization of chimeric Ly-49A and -C receptors suggests that in addition to the carbohydrate recognition domain (CRD) of Ly-49C, part of the stem region is also necessary to confer the class I ligand specificity (25). However, it is not clear whether the requirement for the stem region in determining the receptor specificity is due to the direct involvement of the stem in class I MHC interaction or to the formation of appropriate

[1] To whom correspondence should be addressed. Fax: 301-402-0284. E-mail: sun@magenta.niaid.nih.gov.

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dimers. Much less is known about the structure of human CD94/NKG2 receptors and whether the stem region plays any role in receptor function. To extend our understanding of the physical properties of CD94, we expressed, refolded, and characterized two forms of the extracellular portion of human CD94.

MATERIALS AND METHODS

Expression plasmids and DNA construct. The expression plasmid was derived from a pET30 vector (Novagen, Madison, WI) by first digesting with restriction enzymes *Nde*I and *Nco*I, repairing with Klenow, and then religating to create a pET30Ndel vector which lacks the DNA sequences encoding the 5′ His-tag and S-tag of the parent vector. The fragment encoding the extracellular domain of CD94 (residues 34–179, referred to as S34) was obtained from a baculovirus transfer plasmid pAc-sol94, previously constructed to carry the soluble CD94 receptor (26), by digesting with *Bam*HI and *Eco*RI restriction enzymes. The corresponding cDNA encoding residues 51–179 (referred to as E51) was generated by the polymerase chain reaction (PCR) using a 5′ oligonucleotide primer, 5′-GGGGGATCCGAACTCCAGAAAGACTCTGAC-TGC-3′, and a 3′ oligonucleotide primer, 5′-GGAAG-ATCTGGAAAGGATCAGATCTGCAGCGGC-3′. Both S34 and E51 were subcloned into pET30Ndel using the *Bam*HI and *Eco*RI sites to create the expression plasmids of pCD94S34 and pCD94E51, respectively. Double-stranded sequencing was performed to verify the correct nucleotide sequence.

Strains and media. Plasmids containing the CD94 sequence were transformed into *E. coli* BL21(DE3) cells (Novagen) using Luria-Bertani (LB) agar plates with 100 mg of kanamycin (Sigma, St. Louis, MO). Colonies were screened for the expression of CD94 in 5-mL cultures at 37°C using LB broth (Advanced Biotechnologies Inc., Columbia, MD) containing 50 μg/mL kanamycin and induced with 0.8 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Gold Biotechnologies Inc., St. Louis, MO) after cells had reached an optical density at 595 nm (OD_{595}) of 0.5. The expression level was assessed by the intensity of the CD94 band on a Coomassie blue (PhastGel Blue R, Amersham Pharmacia Biotech, Piscataway, NJ) stained homogeneous 20% polyacrylamide–sodium dodecyl sulfate (SDS) gel (PhastSystem, Amersham Pharmacia Biotech). Glycerol stocks were prepared by adding an equal volume of glycerol to an overnight culture with an OD_{595} of approximately 1.0.

Protein expression, refolding, and purification. A 150-ml overnight inoculant was grown at 37°C using LB broth containing 50 μg/mL kanamycin and with 10 μL of CD94 glycerol stock. This overnight culture was then used to seed a 10-L fermentor vessel (New Brunswick Scientific, Edison, NJ) that contained 9 L of Super-Broth (Advanced Biotechnologies Inc.), 450 mg of kanamycin, and 1 mL of Antifoam 289 (Sigma). The agitation, temperature, and air flow were set at 500 rpm, 37°C, and 15 L/min, respectively, using a Bioflo 3000 Bioreactor (New Brunswick Scientific). The cells were induced with 0.8 mM IPTG upon reaching OD_{595} = 1.5 and harvested after 5 h of induction. Harvested cells were resuspended in a lysis buffer containing 25% sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM Tris–HCl at pH 8.0 and lysed by cycles of freezing, thawing, and sonication. DNAse-I (Sigma) was added to clear away residual chromosomal DNA. Inclusion bodies were collected and washed several times with a wash buffer containing 2 M urea, 5 mM EDTA, 5 mM dithiothreitol (DTT), 0.1% Triton X-100, and 0.1 M Tris–HCl at pH 7.9. The concentrations of the final purified inclusion body were estimated from SDS–polyacrylamide gel electrophoresis (PAGE) (PhastSystem, Amersham Pharmacia Biotech) using Coomassie blue staining.

The reconstitution procedure for both the S34 and E51 constructs of CD94 was the same as that used for crystal structure determination (27). In detail, a 2-L refolding reaction was carried out containing 1 M L-arginine, 0.5 M NaCl, 25 mM $CaCl_2$, 5 mM cystamine, 1 mM NiCl, and 50 mM Tris–HCl at pH 8.0. Approximately 250 mg of CD94 inclusion body was dissolved in 120 mL of 6 N guanidine hydrochloride and injected through a 19-gauge needle in three equal aliquots at intervals of 6 h into the refolding buffer with vigorous stirring. The concentration of dissolved inclusion body protein was approximately 6 μM. The refolding mixture was stirred moderately for 24–48 h at 4°C and then dialyzed (Spectra/Por 3, Spectrum Laboratories Inc., Laguna Hills, CA) against water at 4°C until the final salt content was less than 30 mM, as indicated by a conductivity meter (Model CD-55, Corning Inc., Acton, MA). The dialyzed refolding reaction mixture was centrifuged for 30 min at 27,500*g* (13,000 rpm in a Sorvall GSA rotor) to remove aggregates and then applied to a Mono P column (HR5/20, Amersham Pharmacia Biotech) at a 0.5 mL/min flow rate. The bound CD94 was eluted with a 60-mL elution gradient from 10 mM Hepes, pH 7.0, to 400 mM NaCl, 10 mM Acetate, pH 4.4. Eluted samples were dialyzed (10-kDa Slide-A-lyzer, Pierce, Rockford, IL) against 5 mM Tris–HCl, pH 8.0, further concentrated using a Savant AES1010 SpeedVac (Savant Instruments Inc., Holbrook, NY), and then applied to a Superdex 200 (HR10/30, Amersham Pharmacia Biotech) gel filtration column at a flow rate of 0.25 mL/min with a running buffer containing 0.1 M NaCl and 50 mM Tris–HCl, pH 8.0.

Trypsin treatment. S34 (1.5 mg/mL) was incubated at room temperature in the presence of 0.01 mg/mL porcine modified trypsin (Promega, Madison, WI) and

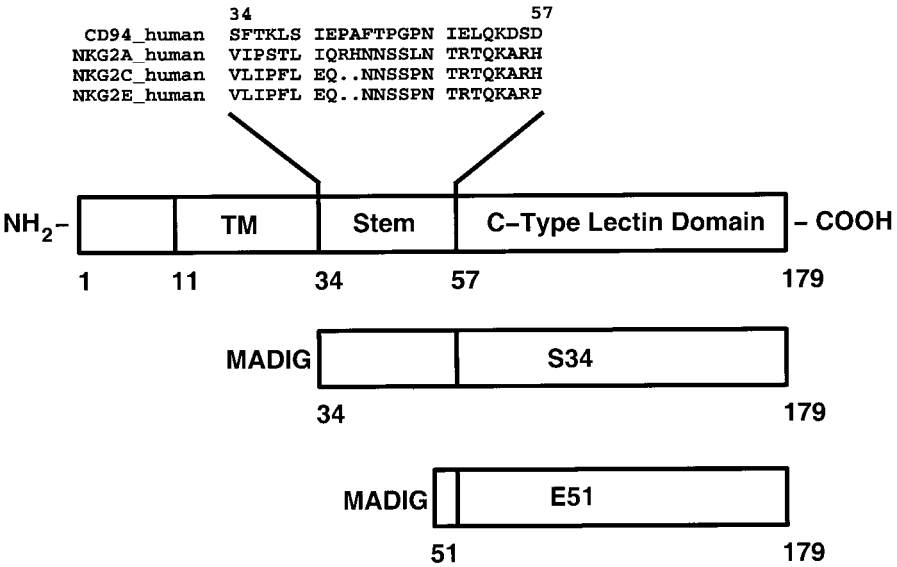

FIG. 1. Domain structure of mature CD94. The numbers indicate amino acid residue position. Expression constructs S34 and E51 are aligned below the mature CD94 structure. The sequence of the human CD94 stem region is shown aligned with stem regions of human NKG2A, -C, and -E.

50 mM Hepes, pH 7.5, for 25 h. The reaction was quenched by adding the serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride HCl (AEBSF, ICN Biomedicals Inc., Costa Mesa, CA) to make a final concentration of 11 μM.

Mass spectrometry and N-terminal amino acid sequencing. Electrospray ionization (ESI) mass spectroscopy (MS) measurements were acquired and recorded with a Perkin–Elmer Sciex API-300 LC/MS/MS system (Perkin–Elmer/Applied Biosystems, Foster City, CA). Protein samples exchanged into a volatile buffer and at a concentration of 10 pM/μL or greater were delivered by direct infusion using an infusion pump in a carrier solution of 30% acetonitrile, 1% acetic acid at a rate of 5 μL/min through a fused-silica capillary of 100-μm internal diameter. When necessary, samples were desalted through an in-line desalting trap cartridge. N-Terminal amino acid sequencing was performed on refolded CD94 by repeated Edman degradation using a Model 477A protein sequencer coupled to a Model 120A PTH analyzer (Perkin–Elmer/Applied Biosystems) at the NIAID core facility. These analyses were done on samples that were purified by gel filtration and concentrated to approximately 5 mg/mL in 50 mM Tris–HCl, pH 8.0.

RESULTS

Protein Expression Constructs and Inclusion Body Preparation

The mature CD94 consists of a short N-terminal intracellular tail (residues 1–10), a single transmembrane region (residues 11–33), and an extracellular region. The extracellular ligand binding domain consists of a 23-residue stem region (residues 34–56) followed by a C-type lectin-like domain (residues 57–179) (Fig. 1). Two extracellular soluble receptor constructs, one with the complete extracellular region (S34) and the other with only the C-type lectin-like domain (E51), were created by PCR and subcloned into a bacterial T7 polymerase based expression system (Fig. 1). A five-residue linker (MADIG) was added to the N-termini of both constructs.

Inclusion bodies of S34 were expressed using a Bioflo 3000 Bioreactor. Nine liters of *E. coli* BL21 culture carrying the expression plasmid was grown to an OD_{595} of 1.5 and induced with 0.8 mM IPTG 5 h before harvest. The yield of S34 inclusion body was estimated to be 75 mg/L (Fig. 2, lanes 2 and 3). E51 was expressed in a 1-L culture flask using growth kinetics similar to that of S34 and the yield of inclusion body was about 100 mg/L (Fig. 2, lanes 4 and 5). The final inclusion bodies after repeated washes with 2 M urea and 0.1% Triton X-100 solution were at least 80% pure.

Refolding of S34 and E51

In brief, purified CD94 inclusion body was first dissolved in 6 N guanidine hydrochloride and then diluted 1:11 into a refolding buffer containing arginine and a redox system. The refolded protein was purified to homogeneity using a Mono P column and a Superdex 200 gel filtration column (Fig. 3A). The yield for refolding was approximately 2 mg/L of refolding solution for both

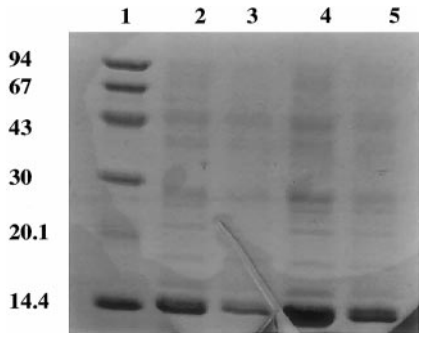

FIG. 2. SDS–PAGE of the S34 and E51 inclusion bodies. A 20% homogeneous gel stained with Coomassie blue was used. Lane 1: low molecular weight standards (Pharmacia Biotech); lane 2: purified S34 inclusion body; lane 3: twofold dilution of lane 2; lane 4: purified E51 inclusion body; lane 5: twofold dilution of lane 4. Molecular weight positions (in kilodaltons) are shown to the left of lane 1.

S34 and E51. The refolded S34 and E51 ran as approximately 17- and 14-kDa bands, respectively, when subjected to SDS–PAGE using a 20% gel (Fig. 3B).

Refolded S34 contains three major species with molecular masses of 17,434, 16,756, and 16,414 Da, respectively, when analyzed by ESI-MS experiments. They correspond to three N-terminal degradation products, starting at amino acid residues ADIGS (full length minus the N-terminal methionine), TKLSIE (residues 36–179), and SIEPAF (residues 39–179), respectively. The molecular weights of all three constructs are consistent with all nine cysteines in oxidized form and having a cystamine adduct on one of the nine cysteines. The mass spectrometry measurement of E51 revealed only one major component with a molecular mass of 14,526 Da. It is consistent with the N-terminal sequence starting at residue Ser 56, the presence of four disulfide bonds, and no cystamine adducts.

Refolded S34 but Not E51 Forms Dimer

A Superdex 200 gel filtration column was used to analyze the multimeric state of S34 and E51. The refolded S34 eluted as a 31-kDa peak and E51 as a 23-kDa peak (Fig. 3A), suggesting that S34 folds into a dimeric form whereas E51 remains monomeric.

Proteolytic Sensitivity and Forming of Interchain Disulfide Bond

The stem region of refolded human CD94 was also observed to be sensitive to trypsin treatment. After incubation of S34 at room temperature in the presence of 0.01 mg/mL trypsin for 25 h, N-terminal sequencing clearly revealed two major species of CD94 starting at residues Leu 38 and Asp 55, both of which are within the stem region.

When the sample of S34 was stored at 4°C for approximately 6 months, further proteolysis and formation of an interchain disulfide bond were both observed. The proteolytic degradation is evident both from SDS–PAGE analysis, showing a lower molecular weight band of S34 (Fig. 4B) compared to the newly refolded sample (Fig. 3B), and from mass spectrometry measurements, showing a species with a molecular mass of 14,689 Da. This molecular mass is consistent with a sequence starting at amino acid residue Asp 55. The formation of an interchain disulfide, thereby producing a homodimeric form, is evident by the appearance of a band at the 29-kDa molecular mass range on a nonre-

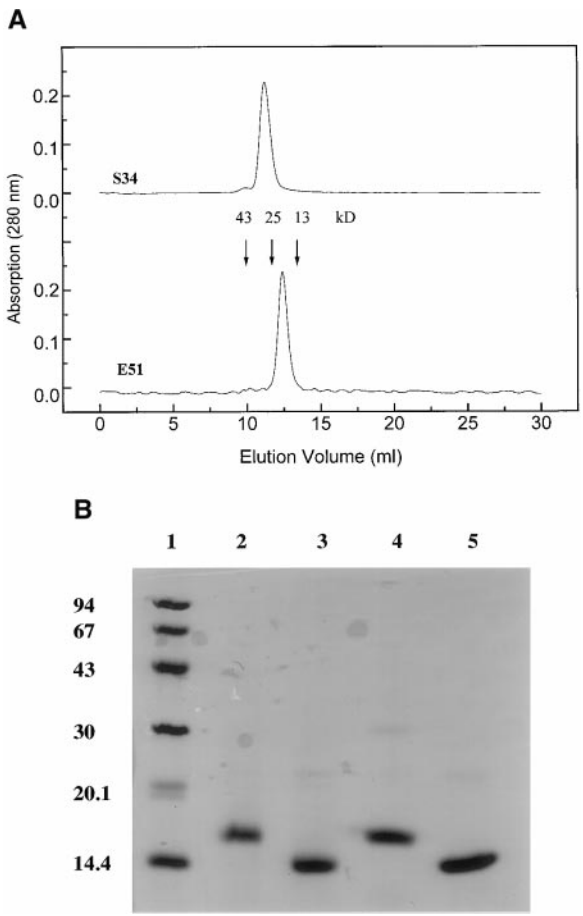

FIG. 3. (A) Superdex 75 gel filtration chromatographs of refolded S34 (top) and E51 (bottom). Chromatographs plot the UV absorbance at 280 nm (*y* axis) against elution volume (*x* axis). Positions of molecular standards (in kilodaltons) are shown between the two chromatographs. (B) SDS–PAGE of refolded S34 and E51 under reduced and nonreduced conditions using a 20% gel stained with Coomassie blue. Lane 1: low molecular weight standards (Pharmacia Biotech); lane 2: S34 under reducing conditions; lane 3: E51 under reducing conditions; lane 4: S34 under nonreducing conditions; lane 5: E51 under nonreducing conditions. Molecular weight positions (in kilodaltons) are shown to the left of lane 1.

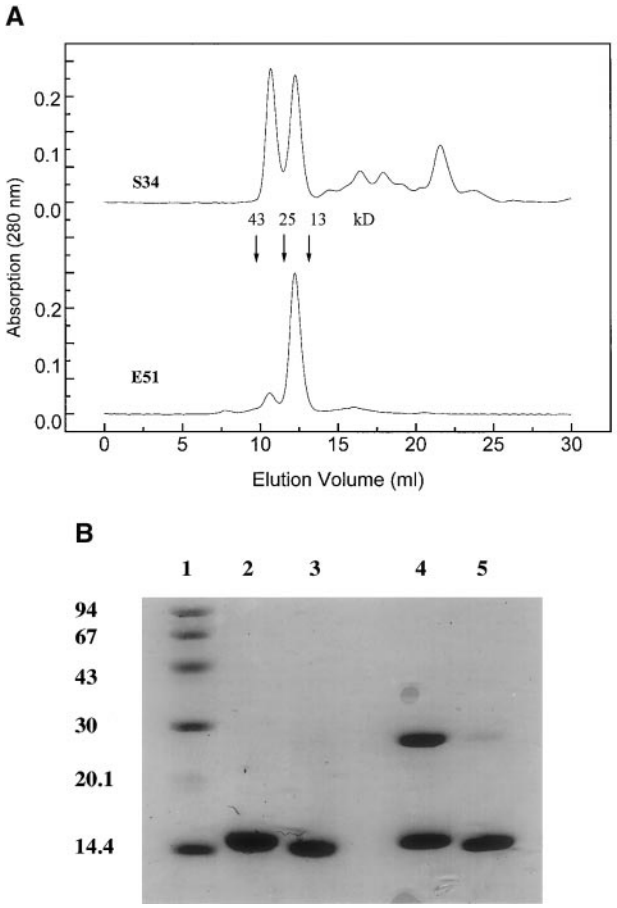

FIG. 4. (A) Superdex 75 gel filtration chromatographs of refolded S34 (top) and E51 (bottom) after several months of storage at 4°C. Chromatographs plot the UV absorbance at 280 nm (*y* axis) against elution volume (*x* axis). Positions of molecular standards (in kilodaltons) are shown between the two chromatographs. (B) SDS–PAGE of refolded S34 and E51 under reduced and nonreduced conditions after extended storage at 4°C. A 20% gel stained with Coomassie blue was used. Lane 1: low molecular weight standards (Pharmacia Biotech); lane 2: S34 under reducing conditions; lane 3: E51 under reducing conditions; lane 4: S34 under nonreducing conditions; lane 5: E51 under nonreducing conditions. Molecular weight positions (in kilodaltons) are shown to the left of lane 1.

ducing SDS gel (Fig. 4B) and by the appearance of a peak at 29,380 in mass spectrometry measurement. This N-terminal degradation product contains an equal mixture of monomeric and dimeric form as analyzed by gel filtration chromatography (Fig. 4A). The monomer to dimer ratio observed in size exclusion chromatography approximately equals the ratio of the disulfide-free form versus the disulfide-bonded form observed in the nonreduced SDS gel, suggesting that the dimeric form of S34 is stabilized by the interchain cysteine bridge. *In vivo,* CD94 is known to form a disulfide-linked heterodimer with NKG2 chain instead of forming a homodimer (5,6). Although there is no direct evidence regarding which cysteines form the CD94/NKG2 heterodimer, our results from this work and the previously published crystal structure of CD94 suggest that the cysteines involved in S34 homodimerization are those mediating the CD94/NKG2 heterodimer formation. This also implies that the role of the interchain cysteine bridge is to stabilize the receptor heterodimer on the cell surface.

In contrast to S34, E51 showed no obvious degradation upon storage for several months. The mass spectrometry result shows the molecular mass of E51 upon storage to be 14,530 Da, nearly identical to that of the freshly refolded E51 sample. However, approximately 10% of this E51 forms a disulfide-linked dimer, as evident both from the nonreducing SDS–PAGE and from the gel filtration chromatography (Figs. 4A and 4B).

DISCUSSION

It is worth noting that the extracellular domain of CD94 contains nine cysteines, forming four intrachain disulfide bonds and one interchain disulfide bond with NKG2 in the functional heterodimer. This precluded the use of a bacterial expression system to obtain soluble, functional receptor proteins. To date, most CD94 expression systems are either cell surface transfection-based using mammalian cells or the baculovirus-based expressing systems. We used a refolding system to obtain the properly refolded extracellular portion of human CD94 from bacterial inclusion bodies. Since CD94 without NKG2 does not recognize the HLA-E ligand, there is no functional binding assay available to directly test the activity of the refolded protein. However, the biophysical properties of the refolded CD94, as judged by gel filtration chromatography, mass spectroscopy, and proteolysis, are consistent with the refolded CD94 having a native structure. This is further supported by the fact that S34 can be crystallized and its structure resembles the folded form of C-type lectin (27).

Gel filtration results show that the stemless E51 protein has much less of a tendency to form dimers than does S34, suggesting an important role for the stem region in dimerization of CD94. However, the proteolytic sensitivity of the S34 stem region suggests that it may be flexible.

It is conceivable that the stem region of CD94 is also involved in the heterodimer formation with that of NKG2 molecules. Interestingly, the stem region of CD94 is mostly negatively charged and the corresponding region of NKG2 is positively charged, with the location of charges appearing to complement each other (Fig. 1). This apparent charge complementarity

could result in favorable interactions between the two stem regions and thus preferentially stabilize the heterodimer over either CD94 or NKG2 homodimers. This is consistent with the observation that the cell surface expression of heterodimers is better than that of each chain alone.

The stem region in both CD94 and NKG2 is short and contains about 20 residues compared to the stem region of Ly-49 molecules, which has about 70 residues. However, by making different chimeric constructs, Brennan *et al.* (1996) showed that only the 20–30 residues immediately adjacent to the CRD of Ly-49A and -C were important for class I MHC recognition. Since the equivalent stem region in CD94 appears to be involved in the dimerization of CD94, it is likely that these regions in Ly-49 are also involved in the homodimer formation of Ly-49 molecules. It is therefore possible that the stem region in Ly-49 molecules is necessary to preserve the appropriate dimer formation for class I MHC recognition and mutations which disrupt the dimerization region may result in altered dimer formation and thus the MHC recognition.

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